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Modelling and experimental analysis of hormonal crosstalk in *Arabidopsis*

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An important question in plant biology is how genes influence the crosstalk between hormones to regulate growth. In this study, we model *POLARIS* (*PLS*) gene function and crosstalk between auxin, ethylene and cytokinin in *Arabidopsis*. Experimental evidence suggests that PLS acts on or close to the ethylene receptor ETR1, and a mathematical model describing possible PLS–ethylene pathway interactions is developed, and used to make quantitative predictions about PLS–hormone interactions. Modelling correctly predicts experimental results for the effect of the *pls* gene mutation on endogenous cytokinin concentration. Modelling also reveals a role for PLS in auxin biosynthesis in addition to a role in auxin transport. The model reproduces available mutants, and with new experimental data provides new insights into how PLS regulates auxin concentration, by controlling the relative contribution of auxin transport and biosynthesis and by integrating auxin, ethylene and cytokinin signalling. Modelling further reveals that a bell-shaped dose–response relationship between endogenous auxin and root length is established via PLS. This combined modelling and experimental analysis provides new insights into the integration of hormonal signals in plants.
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Introduction

Hormone signalling systems coordinate plant growth and development through a range of complex interactions. The activities of plant hormones, such as auxin, ethylene and cytokinin, depend on cellular context and exhibit interactions that can be either synergistic or antagonistic. An important question about understanding these interactions is how genes act on the crosstalk between hormones to regulate plant growth. It is known, for example, that auxin and cytokinin can each induce ethylene biosynthesis (Vogel *et al*, 1998), and ethylene can induce PIN protein expression, auxin transport and auxin biosynthesis in the root (Ruzicka *et al*, 2007; Stepanova *et al*, 2007; Swarup *et al*, 2007), as well as inhibit auxin transport in the stem (Suttle, 1988; Chilley *et al*, 2006). Furthermore, auxin can inhibit cytokinin synthesis (Nordstrom *et al*, 2004).

Previously, we identified the *PLS* gene of *Arabidopsis*, which transcribes a short mRNA encoding a 36-amino acid peptide that is required for correct root growth and vascular development (Casson *et al*, 2002). Experimental evidence shows that there is a link between *PLS*, ethylene signalling, auxin homeostasis and microtubule cytoskeleton dynamics (Chilley *et al*, 2006). Specifically, mutation of *PLS* results in an enhanced ethylene-response phenotype, defective auxin transport and homeostasis and altered sensitivity to microtubule inhibitors. These defects, along with the short-root phenotype, are suppressed by genetic and pharmacological inhibition of ethylene action. The expression of *PLS* is itself repressed by ethylene and induced by auxin. It was also shown that *pls* mutant roots are hyper-responsive to exogenous cytokinins and show increased expression of the cytokinin inducible gene, *ARR5/IBC6*, compared with the wild type (Casson *et al*, 2002). Therefore, *PLS* may also be required for correct auxin–cytokinin homeostasis to modulate root growth.

Thus to make appropriate decisions for growth and development, plant cells must process and integrate the signalling of multiple inputs. Complexity in signalling systems presents serious challenges to understand how cells respond to multiple hormones. In general, one of the requirements to

meet these challenges is the development and application of mathematical models (Komarova *et al*, 2005; Diaz and Alvarez-Buylla, 2006; Schaber *et al*, 2006; Bardwell *et al*, 2007; Zou *et al*, 2008). To quantitatively understand and predict the roles of the crosstalk between auxin, ethylene and cytokinin signalling in plant growth, this study develops a mathematical model for the action of the *PLS* gene on the crosstalk between auxin, ethylene and cytokinin signalling in *Arabidopsis*.

A combined modelling and experimental analysis is applied in this study. First, existing literature relating to the action of *PLS* on the crosstalk between auxin, ethylene and cytokinin is mapped to a mathematical model. The interaction point between the PLS peptide and ethylene signalling has not been identified biochemically, although there is strong genetic evidence to suggest that PLS acts at the ethylene receptor (Chilley *et al*, 2006). We analyse the possibilities for the interactions between the PLS peptide and ethylene receptor and identify the one leading to correct response phenotypes. In addition, analysis of experimental data describing the regulation of *PLS* transcription by auxin and ethylene reveals that *PLS* regulation by ethylene is independent of auxin, although ethylene also regulates auxin biosynthesis.

On the basis of these analyses, a model is subsequently developed and used to make predictions that are tested experimentally. The results describe the possibilities for a role for the PLS peptide at the ethylene receptor and CTR1 kinase, and they provide new evidence for a link with cytokinin signalling; and for a role for PLS in auxin transport and biosynthesis, to regulate auxin accumulation in the root tip. Model analysis reveals that a bell-shaped dose–response relationship between endogenous auxin and root length is established via PLS to regulate root growth rate.

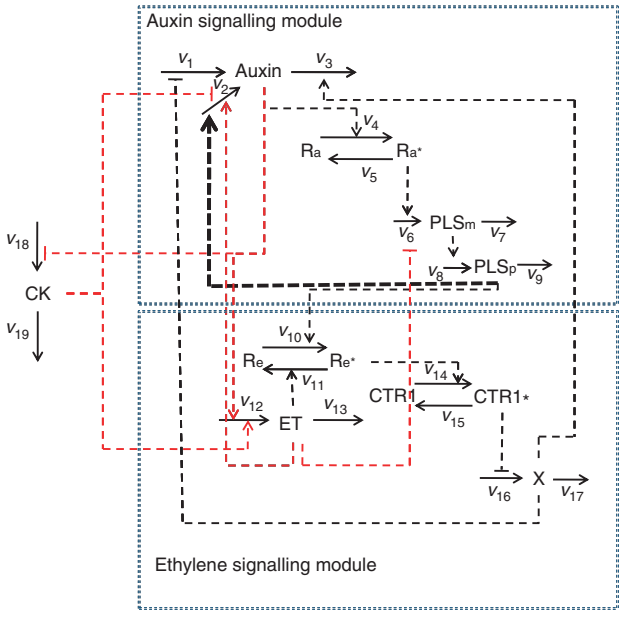


Figure 1 Model for the action of *POLARIS* gene on the crosstalk of auxin, ethylene and cytokinin in *Arabidopsis*. Solid arrows indicate conversions, whereas dotted lines indicate regulations. Black dotted lines indicate regulation through auxin, ethylene or CTR1 receptors, whereas red dotted lines indicate these interactions, for which the molecular basis is unknown, but biological evidence shows their existence. There are a number of different possible ways to represent these interactions. For simplicity, we have included them without further expanding their interactions with the relevant receptors. The wide dotted line indicates regulations that are revealed by the discrepancy in auxin concentration in *pls* between modelling and experimental results (see Model predictions and experimental measurements section for details).

Results

Model development

A model for crosstalk between auxin, ethylene and cytokinin via the *PLS* gene was constructed on the basis of known molecular interactions and experimental evidence. The model is shown in Figure 1 and in Supplementary information. Model equations and parameter values are included in the Supplementary information. The model is a single-cell model applied to root development in *Arabidopsis*. The model construction process is as follows:

Auxin signalling crosstalk

In the model, two sources of auxin can be considered: auxin biosynthesis within the cell and auxin transport from other cells. Auxin enters the cell both due to passive permeability (diffusion) and by the action of influx carriers (AUX/LAX family; Bennett *et al*, 1996; Ljung *et al*, 2001). Experimental evidence (Suttle, 1988; Chilley *et al*, 2006) indicates that a relatively high ethylene signalling response inhibits the transport of auxin from the shoot to the root tip. However, the molecular basis of this inhibition is unclear. We assume that a molecule or molecules, *X*, located downstream of ethylene signalling, inhibit the transport of auxin from shoot to root (see 'Ethylene signalling crosstalk' below for more details). It is also evident that ethylene activates the biosynthesis of auxin locally in the root tip (Stepanova *et al*, 2007; Swarup *et al*, 2007), and it is known that cytokinin inhibits subsets of auxin responses (including hypocotyl elongation and lateral root growth; Casson and Lindsey, 2003; Coenen *et al*, 2003) and the biosynthesis of auxin (Eklof *et al*, 1997).

By taking these factors into account, we use two rate equations (v_1 and v_2) to describe the sources of auxin. In v_2, a background synthetic rate, k_2, is included, and it represents the auxin biosynthetic rate that is independent of ethylene and cytokinin. v_2 stands for the case in which ethylene and cytokinin simultaneously act on a single pathway for auxin biosynthesis. In the Supplementary information, different types of kinetics for auxin biosynthesis and their relation to different reaction schemes are examined in detail. Auxin leaves the cell via efflux carriers (PINs and PGPs; Geislera and Murphy, 2006; Petrasek *et al*, 2006). In addition, the removal of biologically active free auxin also occurs by transport out of the cell and by conjugation or degradation (LeClere *et al*, 2002; Qin *et al*, 2005). It is assumed that the loss of active auxin follows $v_3=(k_3+k_{3a}[X])[\text{auxin}]$, where $k_3[\text{auxin}]$ describes degradation/conjugation; and $k_{3a}[X][\text{auxin}]$ describes the ethylene-mediated upregulation of PINs (e.g. PIN2) via its downstream response, *X*, to remove auxin from the more distal region of the root tip (Ruzicka *et al*, 2007).

The auxin signalling response and its relationship with the *PLS* gene is described as follows: auxin binds with the inactivated form of its receptor, Ra, and changes it into the activated form, Ra*, with mass–action kinetics, v_4; the conversion of the activated form into the inactivated form follows first-order kinetics, v_5. The total auxin receptor concentration is [RaT]=[Ra] + [Ra*]. The effects of varying total auxin receptor concentration are analysed in the Supplementary information. Ra* subsequently activates downstream gene expression that includes *PLS* gene transcription. The rate for *PLS* transcription depends on [Ra*], reflecting the auxin-mediated activation of *PLS* (Casson *et al*, 2002). The details of PLS translational control are not clear, and therefore its rate is simply described by first-order kinetics. The degradation of *PLS* mRNA (PLSm) and protein (PLSp) is also included in the model.

Ethylene signalling crosstalk

It is known that both auxin and cytokinin can synergistically activate the biosynthesis of ethylene (Vogel *et al*, 1998; Stepanova *et al*, 2007). However, ethylene can also be synthesized without exogenous auxin and cytokinin application, such as in its role in root hair production (Tanimoto *et al*, 1995); and several studies place auxin signalling downstream of ethylene signalling (e.g. Roman *et al*, 1995; Stepanova *et al*, 2005; Chilley *et al*, 2006; Ruzicka *et al*, 2007; Swarup *et al*, 2007). Therefore, we can consider ethylene synthesis as comprising two components, as shown in v_{12}. The first is a background synthetic rate, k_{12}, which will be considered a constant. The second includes the activation of ethylene biosynthesis by both auxin and cytokinin. In the Supplementary information, different types of kinetics for ethylene biosynthesis and their relation to different reaction schemes are examined. Removal of ethylene follows simple first-order kinetics.

Following Diaz and Alvarez-Buylla (2006), ethylene signalling can be described as follows: ethylene acts on the activated form of its receptor, Re*, and changes it into the inactivated form, Re, with mass–action kinetics depending on both [ET] and [Re*], v_{11}. This ethylene–receptor interaction then leads to a loss of interaction between the receptor complex and the negative regulator MAP kinase, CTR1, and ethylene responses then occur. Receptor deactivation follows first-order kinetics. The total concentration of the receptor, ReT, is assumed to be conserved, [ReT]=[Re] + [Re*]. The activated ethylene receptor Re* activates CTR1, with mass-action kinetics, v_{14}. The activated form of CTR1, CTR1*, is deactivated with first-order kinetics. The total CTR1 concentration, CTR1T, is assumed to be conserved, and represented as [CTR1T]=[CTR1] + [CTR1*]. The effects of varying ReT and CTR1T are analysed in the Supplementary information. The active CTR1* subsequently activates a MAPK cascade, and activation of ethylene response requires inactivation of this MAPK cascade (Diaz and Alvarez-Buylla, 2006). Once the MAPK cascade is blocked by the presence of ethylene, EIN2 is activated, and expression of *ERF1* and its downstream targets are activated (not shown in Figure 1). Activation of EIN2 depends on the concentration CTR1*. If CTR1* is small, the EIN2-activated form is large, and as [CTR1*] increases, the EIN2-activated form decreases. Therefore, CTR1* has an inhibitory role in the activation of the downstream ethylene signalling response.

As the molecule(s) in the downstream ethylene signalling response, which interact with the auxin signalling module, are largely unknown, the model does not, for the sake of simplicity, explicitly include the MAPK cascade and the pathway downstream from EIN2. Instead, we use a 'component X' to represent the ethylene-regulated molecule(s) that interact with auxin signalling. The production of X is inhibited by the activated CTR1*, as explained above. The rate for X production is $v_{16}=k_{16}-k_{16a}$[CTR1*] and the values of k_{16} and k_{16a} are selected to reflect the following two facts: (a) if [CTR1*]=[CTRT], that is, all CTR1 molecules are in the activated state, ethylene signalling response is not activated; and (b) as CTR1* concentration decreases, the ethylene signalling response increases.

Analysis of interactions between PLS and ethylene signalling

Experimental evidence supports the view that the interaction between the PLS peptide and ethylene signalling module is at or close to the ethylene receptor ETR1 (Chilley *et al*, 2006), although how PLS protein may interact at the molecular level with ETR1 or with other components of the ethylene signalling pathway is not clear. Here, in the following text and in Supplementary information, we examine all possibilities of the PLS peptide interacting with ETR1 and CTR1 proteins to determine which interactions qualitatively lead to experimentally observed results.

If the PLS protein interacts with the ethylene receptor, with a function of activating the changes from the inactive form of ethylene receptor, Re, to its activated form, Re*, the kinetics of the ethylene receptor and CTR1 protein are described as follows:

$$\begin{aligned}\frac{d[Re*]}{dt} &= v_{10} - v_{11}\\ \frac{d[CTR1*]}{dt} &= v_{14} - v_{15}\end{aligned} \tag{1}$$

At a steady state,

$$[Re*] = \frac{[ReT]}{1 + \frac{k_{11}[ET]}{k_{10}+k_{10a}[PLSp]}} \tag{2}$$

$$[CTR1*] = \frac{[CTR1T]}{1 + \frac{k_{15}}{k_{14}[ReT]}\left(\frac{k_{11}[ET]}{k_{10}+k_{10a}[PLSp]} + 1\right)} \tag{3}$$

The relationship between [PLSp], [Re*] and [CTR1*] reveals the following scenario. If [PLSp] decreases, both [Re*] and [CTR1*] decrease. As CTR1* negatively regulates the downstream ethylene response, X, there is an increase in X. This, in turn, leads to a lower transport rate of auxin from shoot to root and, therefore, to a lower auxin concentration in the root. The trend qualitatively agrees well with experimental observations: the *pls* mutant has a lower transport rate of auxin from shoot to root, a lower root auxin concentration and a shorter root length (Chilley *et al*, 2006). Moreover, Chilley *et al* (2006) observed that for [PLSp]=0 μM (i.e., the *pls* mutant), reducing the functional interaction between ethylene and the ethylene receptor by genetic or pharmacological methods restores wild-type auxin

concentration in the root. In Equation (3), this corresponds to the decrease in k_{11}. Equation (3) predicts that if k_{10} is not zero, decreasing k_{11} increases [CTR1*]. Consequently, transport rate of auxin from shoot to root is higher, leading to the increase in auxin concentration. Therefore, the lower auxin concentration of the *pls* mutant can be rescued in the model by reducing ethylene responses, as observed experimentally (Chilley *et al*, 2006). However, if k_{10}=0 s^{-1}, Equation (3) shows that k_{11} no longer affects [CTR1*] for the *pls* mutant, and therefore it no longer affects auxin concentration. As this result contradicts experimental observations, k_{10} should therefore not be zero. The result implies that inter-conversion between the inactivated and activated forms of the ethylene receptor occurs, regardless of whether the PLS peptide acts on the ethylene receptor. Importantly, however, PLS more effectively changes the inactivated form to the activated form, regulating the ethylene signalling response. This analysis shows that the above-proposed interaction is a candidate for explaining experimental observations.

This kind of analysis has been applied to all possible interactions between PLSp and the ethylene receptor. It was found that if PLS protein inhibits the conversion of Re* to Re, this interaction is equivalent to the interaction analysed above, and it is also a candidate for explaining experimental observations (see Supplementary information for details). Therefore, there are two qualitatively equivalent candidates for explaining experimental observations when PLSp interacts with the ethylene receptor. However, all other interactions with the receptor cannot lead to the results that agree with experimental observations. For example, these interactions include PLSp activating the conversion from Re* to Re; PLSp binding with Re* and forming another inactive state; or PLSp binding with Re and forming another inactive state. Therefore, these possibilities are discarded (see Supplementary information for further analysis).

Given that the PLS peptide has a predicted endoplasmic reticulum (ER) retention signal (Casson *et al*, 2002), consistent with the location of the ethylene-binding face of the ethylene receptor protein ETR1 (Chen *et al*, 2002), we favour a model in which PLSp interacts with the ethylene receptor, Figure 1.

Therefore, we have two candidates for the interaction between the PLS peptide and ethylene signalling module at or close to ETR1/the ethylene receptor: (a) PLSp activates the conversion of Re into Re* and (b) PLSp inhibits the conversion of Re* into Re. The two candidates qualitatively lead to the same experimental observations. To distinguish between the two candidates, further quantitative experiments are required to determine kinetic parameters for the two interactions. As the kinetic parameters for those interactions are unknown, it is not possible to distinguish the two candidates at this stage. However, two candidates for the interaction between PLSp and ethylene receptor lead to the same results: namely PLSp increases Re* (see Supplementary information for details). We study the network shown in Figure 1 in detail.

Dependence of *PLS* transcription on auxin and ethylene

Experimental observations show that auxin positively regulates *PLS* transcription (Casson *et al*, 2002) and ethylene negatively regulates *PLS* transcription (Chilley *et al*, 2006). It is also evident that ethylene can activate the biosynthesis of auxin locally (Stepanova *et al*, 2007; Swarup *et al*, 2007). In wild type, increase in ethylene level increases auxin synthesis and transport to the root elongation zone, which thereby inhibits root-length elongation. As ethylene activates the biosynthesis of auxin locally and auxin positively regulates *PLS* transcription, the negative regulation of ethylene on *PLS* transcription suggests that, in addition to the role of ethylene in the biosynthesis of auxin, ethylene must also regulate *PLS* transcription independently of auxin.

The auxin-mediated mechanism regulating *PLS* transcription is not well understood, though the *PLS* gene promoter contains auxin-response elements, suggesting ARF-mediated activation (Casson *et al*, 2002). From a modelling point of view, there are a number of ways to include the negative regulation of ethylene on *PLS* transcription. For example, ethylene may potentially lead to the conversion of the active auxin receptor to an inactive form, or may positively affect Aux/IAA interactions with ARF transcription factors (including those that may lead to transcriptional activation of the *PLS* gene), among other possibilities. Alternatively, other, currently unidentified, ethylene-regulated proteins may act as transcriptional repressors of *PLS* transcription. From a modelling point of view, these possibilities have the same effects. We assume for the purpose of simplicity that ethylene directly affects *PLS* transcription, ν_6, independently of auxin regulation.

Rate equations and parameters

We have tested the applications of both mass–action kinetics and other types of kinetics (e.g. Michaelis–Menten kinetics), finding that different types of kinetics lead to similar results after parameter values are adjusted. Therefore, to reduce the number of parameters, we use mass–action kinetics wherever it is possible.

Some parameters relating to ethylene receptors and CTR1 (i.e. k_{10}, k_{11}, k_{14} and k_{15}) were studied by Diaz and Alvarez-Buylla (2006), and we use the same parameter values for those rates as they did. Other parameters are unknown. Initially, all unknown parameters are set to be unity. Subsequently, a small number of parameters are adjusted as follows:

First, a relatively high ethylene signalling response inhibits the transport of auxin from the shoot to the root tip (Suttle, 1988; Chilley *et al*, 2006) and increases auxin removal from the root tip (Ruzicka *et al*, 2007; Swarup *et al*, 2007), and ethylene activates the biosynthesis of auxin locally (Stepanova *et al*, 2007; Swarup *et al*, 2007). Therefore, ethylene has a dual role in regulating endogenous auxin levels (i.e. through effects on auxin transport into and out of the root, and biosynthesis in the root). If ethylene effects on auxin transport dominate, the increase in endogenous ethylene decreases endogenous auxin in the root tip, through decreased import and increased export. However, if the effects on the biosynthesis of auxin are more dominant, increase in endogenous ethylene level increases endogenous auxin. Figure 2 shows the dependence of auxin concentration on exogenous 1-aminocyclopropane-1-carboxylic acid (ACC) concentration, which increases endogenous ethylene, for two different values of the rate constant for biosynthesis of auxin, k_{2a}.

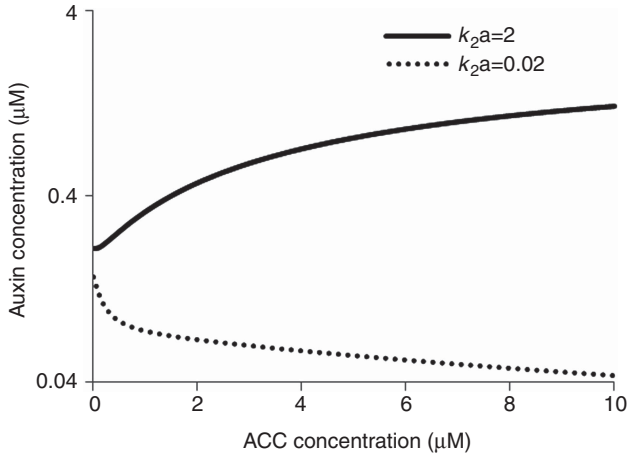


Figure 2 Dependence of auxin concentration on exogenous ACC concentration for two different values of rate constant for auxin biosynthesis, k_{2a}, shows that different values of rate constant, k_{2a}, can result in opposite trends.

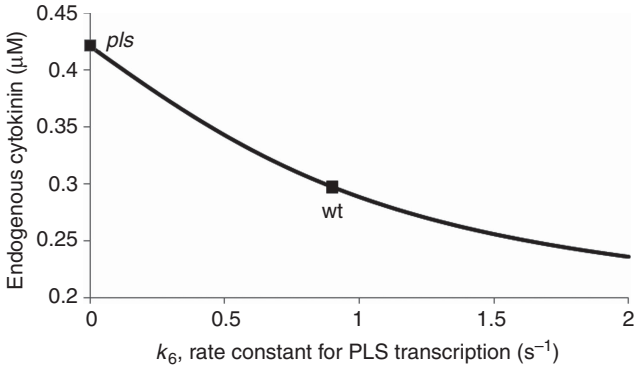


Figure 3 Model prediction for endogenous cytokinin concentration in wild type and *PLS* mutant is in agreement with experimental observations.

When k_{2a} is small ($0.02\,s^{-1}$), increase in ACC concentration decreases auxin concentration. When k_{2a} is large ($2\,s^{-1}$), increase in ACC concentration increases auxin. As different k_{2a} values can lead to opposite trends, the choice of k_{2a} values is important for modelling results. It is evident that in wild type (Stepanova *et al*, 2007; Swarup *et al*, 2007), increase in ACC concentration increases auxin concentration. Therefore, we choose k_{2a}=$2.8\,s^{-1}$so that modelling results are in agreement with experimental observations.

Second, parameter values are further adjusted so that the auxin concentration in *pls* and in wild type is 0.14 and 0.23 μM, respectively, corresponding to experimental observations (Chilley *et al*, 2006). The set of parameters corresponding to wild type are listed in Supplementary information, and they are used to analyse the system throughout this study without further adjustment. In *pls*, k_6=$0\,s^{-1}$. Numerical tests reveal that the model developed in this study (Figure 1 and parameters in Supplementary information) is able to establish a steady state within 50 s in general, when different initial conditions are applied (see Supplementary information for details). In the following text, all computational results are obtained at steady states.

Model predictions and experimental measurements

We used the model to examine mutants known to link the action of PLS to the crosstalk between auxin and ethylene. The model is able to reproduce quantitatively all relevant mutants available, as described in the following sections. Therefore, we consider that the model has properly included known molecular interactions and experimental evidence relating to the action of PLS. We describe here how we used the model to make predictions and design new experiments to investigate PLS function.

Model prediction and experimental measurements of endogenous cytokinin concentration are in agreement

It is known that auxin can negatively regulate cytokinin biosynthesis (Nordstrom *et al*, 2004). The accumulated level of cytokinin is simply described by the balance between its biosynthesis and its removal. To predict the action of PLS in cytokinin signalling, this pathway has been incorporated into the auxin–ethylene model discussed above.

Figure 3 predicts that increase in *PLS* transcription decreases the endogenous cytokinin concentration. It is predicted that in the *pls* mutant, endogenous cytokinin concentration is increased to 1.48-fold of that in wild type. To experimentally test this prediction, the concentrations of endogenous cytokinins were measured using GC-MS in both *pls* and wild-type seedlings.

The analysis of whole seedlings at 10 days post-germination shows that the concentrations of ZMP, iPMP, ZR, Z7G and Z9G were significantly increased in *pls* compared with wild type (Table I). It can be seen that different cytokinins have significantly different fold changes. However, the general trend is that endogenous cytokinin levels in the *pls* mutant are significantly increased, with a median of the fold change being 1.42. This trend is qualitatively predicted by the model, reflecting that the model is able to analyse crosstalk between auxin, ethylene and cytokinins.

In addition, on the basis of our model (Figure 1), one prediction on the interplay between cytokinin, auxin, ethylene and PLS is that the role of cytokinin in regulating PLS transcription is mediated through the ethylene and auxin pathways. Specifically, as cytokinin negatively regulates the biosynthesis of auxin and positively regulates ethylene biosynthesis, increase in endogenous cytokinin concentration decreases endogenous auxin concentration and increases endogenous ethylene concentration simultaneously. Moreover, on the basis of experimental observations that auxin positively regulates *PLS* transcription (Casson *et al*, 2002) and ethylene negatively regulates *PLS* transcription (Chilley *et al*, 2006) and as summarised in our model (Figure 1), cytokinin should negatively regulate *PLS* transcription. Our experiments (Figure 5A in Casson *et al*, 2002) have previously shown that cytokinin indeed negatively regulates *PLS* transcription in the presence of 10 μM kinetin, a cytokinin. Therefore, our model (Figure 1) is consistent with all available experimental evidence for the control of *PLS* transcription by cytokinin, auxin and ethylene.

Table I Cytokinin content (ng/gFW) in wild-type and *pls* mutant seedlings

	iPA	ZOG	ZROG	DHZR	iP	Z	iPMP	ZR	Z7G	Z9G	ZMP	DHZ
wt	0.80	6.80	1.65	0.18	0.14	0.24	9.93	4.33	21.79	8.06	13.68	ND
s.d.	0.18	0.43	0.11	0.02	0.02	0.05	1.47	0.31	2.02	0.83	1.83	
pls	0.72	7.14	1.92	0.21	0.18	0.34	14.94	7.50	38.16	14.86	47.30	ND
s.d.	0.11	1.57	0.40	0.02	0.02	0.06	2.73	0.85	4.75	2.94	5.04	
pls/wt	0.90	1.05	1.16	1.17	1.29	1.42	1.50	1.74	1.75	1.84	3.46	

DHZ, dihydrozeatin; DHZR, dihydrozeatin riboside; iP, isopentenyladenine; iPMP, isopentenyladenine-5′-monophosphate; Z, zeatin; Z7G, zeatin-7-glucoside; Z9G, zeatin-9-glucoside; ZMP, zeatin riboside-5-monophosphate; ZOG, zeatin-*O*-glucoside; ZR, zeatin riboside; ZROG, zeatin riboside-*O*-glucoside.
s.d. represents the standard deviation of the means of five independent biological replicate assays. *pls*/wt represents the ratio of cytokinin content in the *pls* vs wild-type seedling.

Discrepancy in auxin concentration in *pls* between modelling and experimental results reveals a role of *PLS* in auxin biosynthesis

Figure 4A predicts that the exogenous application of ACC to wt, *pls* and *PLSox* seedlings would lead to an increased concentration of endogenous auxin. It has been experimentally shown that for wild type, endogenous auxin concentration increases with exogenously applying ACC (Stepanova *et al*, 2007; Swarup *et al*, 2007). Therefore, the model has correctly predicted this trend for wild-type plants.

To experimentally test the prediction in Figure 4A, the auxin response of the *pls* mutant to exogenous ACC application was investigated. A DR5::GFP reporter, which monitors auxin concentration/response (Sabatini *et al*, 1999), was introduced into the *pls* mutant and, for comparison, wild-type plants and seedlings were grown in the presence of 10 μM ACC for 5 days before analysis.

The results show that, as expected (Ruzicka *et al*, 2007; Swarup *et al*, 2007), ACC treatment led to an increased DR5 signal in the wild-type root tip (Figure 4B). However, for *pls*, the DR5::GFP signal did not increase after ACC treatment. This result is in conflict to the model prediction. This discrepancy indicates that PLS must be required for correct DR5::GFP activation by ACC/ethylene, suggesting an additional role for PLS in regulating either ethylene-induced auxin biosynthesis, auxin accumulation or auxin responsiveness (perception or signal transduction), which was not included in the model.

The model was therefore modified, taking into account the following considerations: when ACC is exogenously applied, the trend for the change in endogenous auxin concentration/response for *pls* (Figure 4B) and wild-type is opposite. Equation (4) shows the dependence of endogenous auxin concentration/response on endogenous ethylene concentration, which increases when ACC is exogenously applied.

$$[\text{auxin}] = \frac{v_1 + v_2}{k_3 + k_{3a}[X]} = \frac{\frac{k_{1a}}{1+[X]/k_1} + k_2 + \frac{k_{2a}[\text{ET}]}{(1+[\text{CK}]/k_{2b})}}{k_3 + k_{3a}[X]} \qquad (4)$$

In wild type, increase in exogenous ACC availability increases the downstream response component, X, and decreases the transport rate of auxin to the root, v_1. To increase the endogenous auxin concentration in wild type relative to that for [ACC]=0 in wild type, v_2 must increase in such a way that the effect of both increases in X and decreases in v_1 is more than compensated by the increased v_2. This requires the rate

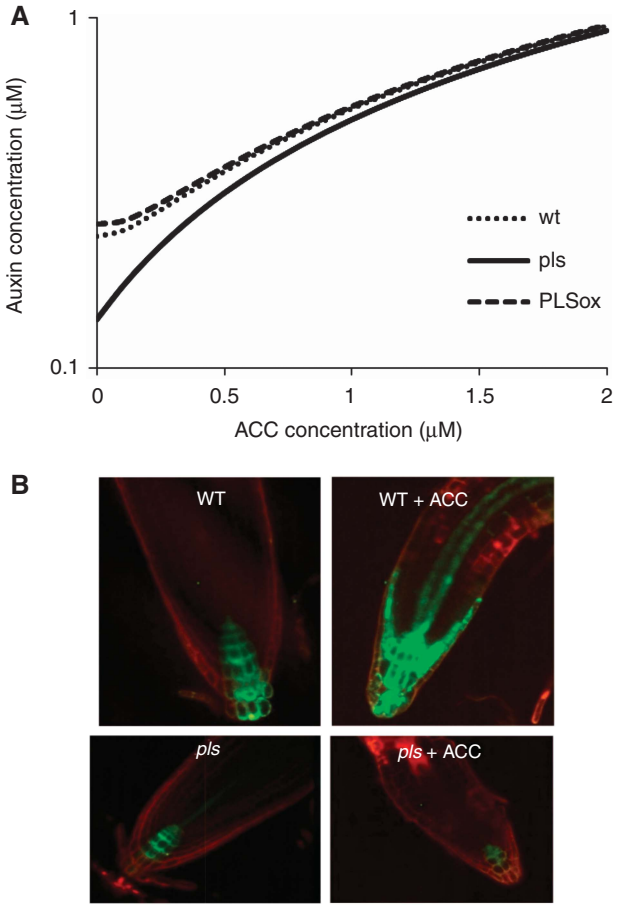


Figure 4 Discrepancy of auxin concentration in *pls* between modelling and experimental results reveals a role of *PLS* in auxin biosynthesis (see text for details). (**A**) Model prediction of auxin concentration in *pls*, wt and PLSox in response to the application of the ethylene precursor ACC; (**B**) Experimental demonstration that ACC induces auxin response in wild-type root tips (revealed as DR5::GFP expression; upper two panels), but the *pls* mutant shows no DR5::GFP induction by ACC (lower two panels). The *pls* mutant (lower left panel) also shows a reduced DR5::GFP signal compared with wild type (upper left panel) in the absence of ACC treatment, consistent with an experimentally lower auxin concentration in the root tip (Chilley *et al*, 2006).

constant for biosynthesis of auxin to be large, as analysed in section A (Figure 2).

In *pls*, [PLSp]=0. X is approximately a constant when exogenous ACC increases. Following $v_1 = k_{1a}/(1 + [X]/k_1)$, v_1 is approximately a constant as well. In the *pls* mutant, therefore,

neither X nor v_1 has a significant role in reducing auxin concentration or response. We can eliminate the latter possibility because we have previously demonstrated that the *pls* mutant is able to respond to auxin, even at very low (pM) concentrations (Casson *et al*, 2002). Therefore, as ACC concentration increases, the accumulated auxin concentration in *pls* relative to that for [ACC]=0 in *pls* will increase due to increase in v_2; increase in v_2 is to such an extent that auxin concentration in wild type also increases. Numerical calculations have also confirmed that the trend between endogenous auxin concentration and ACC for both wild type and *pls* cannot be opposite for a wide range parameter values tested. Therefore, the biosynthesis rate of auxin has to be modified to accommodate experimental results for wild type and *pls*, respectively.

To accommodate the experimental results for wild type and *pls*, v_2 should have the following property: for a fixed ethylene concentration, decreasing *PLS* expression should decrease v_2. Therefore, PLS needs to have a role in both auxin biosynthesis and transport (contributing to accumulated auxin levels). Accordingly, we modify v_2 into

$$v_2 = k_2 + \frac{k_{2a}[\mathrm{ET}]}{(1+[\mathrm{ck}]/k_{2b})}\frac{[\mathrm{PLSp}]}{(k_{2c}+[\mathrm{PLSp}])} \qquad (5)$$

Equation (5) describes the case in which ethylene, cytokinin and PLSp simultaneously act on a single pathway for auxin biosynthesis. In the Supplementary information, different types of kinetics and their relation to different reaction schemes for auxin biosynthesis are examined in detail. After incorporating Equation (5) into the model, parameters for the accumulation of auxin are adjusted so that for *pls*, the endogenous auxin concentration is 0.14 μM. Due to the regulation of auxin biosynthesis by PLS, the modified model predicts that auxin concentration increases more quickly with increased *PLS* transcription, k_6. Therefore, wild type and PLSox correspond to k_6=0.3 $s^{-1}$and k_6=0.45 s^{-1}, respectively. The new parameters are: k_{2c}=0.01 μM; k_2=0.2 μM s^{-1}. The k_2 value reflects that, for *pls* ([PLSp]=0 μM), there is a background biosynthesis rate for auxin. All other parameters are the same as described in the Supplementary information. The modified model predicts the trend of endogenous cytokinin concentration exactly as shown in Figure 3.

Relationship between PLS and auxin and ethylene signalling

Role of PLS in regulating auxin concentration and response in the root

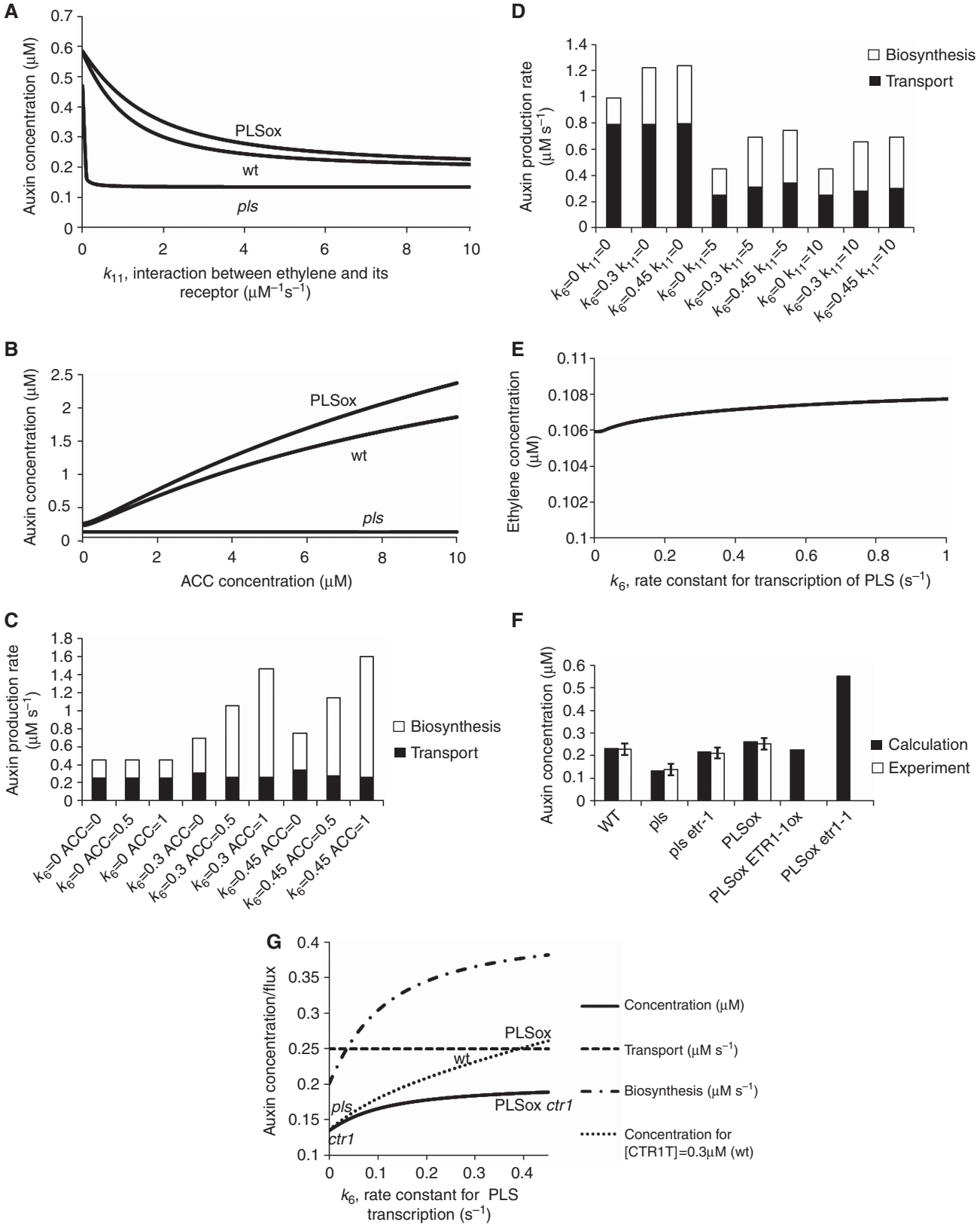
Auxin accumulation in the root derives from two sources: auxin transport from shoot to root and auxin biosynthesis locally at root tip. The rate of removal of active auxin, by transport out of the root or by degradation/conjugation, potentially also affects the concentration in the root tip.

In Figure 5A, we model the effects of both *PLS* transcription, k_6, and the ethylene response through its receptor-signalling pathway, k_{11}. k_{11} can be reduced experimentally by genetic or pharmacological inhibition of ethylene signalling (Chilley *et al*, 2006). Model analysis predicts that interaction of the PLS peptide with the ethylene signalling pathway can flexibly regulate auxin concentration and response. If *PLS* is not expressed (*pls*), the regulation of auxin concentration and its response by the interaction between ethylene and its receptors is less flexible. When k_{11} changes in the range of 0.1–10 $\mu M^{-1} s^{-1}$ (for wt, k_{11}=5 $\mu M^{-1} s^{-1}$), the change in auxin concentration is less than 0.1% (Figure 5A). To change the auxin concentration markedly, k_{11} needs to be reduced to 0.01 $\mu M^{-1} s^{-1}$. However, the prediction described in Figure 5A is that if *PLS* is expressed (in wild type or *PLSox* seedlings), changing k_{11} provides more flexibility in the regulation of auxin concentration/response. In addition, the interaction between PLS and ethylene signalling provides the system with an enhanced capability to regulate auxin concentration, by changing either *PLS* expression, ethylene signalling or both.

Figure 5B predicts that increase in exogenous ACC concentration leads to an increase in endogenous auxin concentration and response in wild type, and that in *pls*, increase in ACC decreases auxin concentration. The prediction of Figure 5B is in agreement with experimental observations (Figure 4B), as experimental analysis in Figure 4B shows that wild-type seedlings expressing *PLS* show a strong induction of DR5::GFP, whereas *pls* mutant shows no induction of DR5::GFP signal by ACC. Therefore, the combined modelling and experimental analysis demonstrates PLS is required for the accumulation of auxin in the root tip in response to ACC/ethylene. In addition, Figure 5C predicts that, in the *pls* mutant, exogenous ACC fails to promote both auxin transport into, and auxin accumulation in, the root (auxin biosynthesis contributes 44% of accumulated auxin levels).

Our model therefore predicts that, in wild type, as ACC increases, the auxin biosynthetic rate increases and the rate of auxin transport into the root decreases; though PIN2 and auxin removal will increase. At a steady state, the effects of ACC in wild type are to increase auxin concentration, as observed experimentally (Stepanova *et al*, 2007; Swarup *et al*, 2007). For [ACC]=0.5 μM, the rate of auxin biosynthesis contributes 75% to the rate of auxin accumulation in wild type. In *PLSox*, ACC is predicted to have a similar effect. As the rates of both auxin transport and auxin biosynthesis are also affected by k_6, Figure 5C clearly shows the dual roles of both k_6 and ACC in auxin transport and auxin biosynthesis.

Figure 5D shows that when k_{11}=0 $\mu M^{-1} s^{-1}$ (i.e. when the ethylene response through its receptor is zero), auxin transport to the tip is more important than auxin biosynthesis in determining auxin concentration (auxin biosynthesis contributes 35% for k_6=0.3 s^{-1}, wild type). Moreover, when k_6, the transcription rate constant of *PLS*, increases, the rate of auxin transport remains unchanged, although the rate of auxin biosynthesis increases (auxin biosynthesis contributes 20% for k_6=0 s^{-1} (*pls*) and 37% for k_6=0.45 s^{-1} (*PLSox*), respectively). Therefore, the increase in auxin concentration after an increase in *PLS* transcription (k_6) is predicted to be due to an increase in auxin biosynthesis. As k_{11} increases (in which case the ethylene response through its receptor is more active), auxin transport becomes less important (auxin biosynthesis contributes 55% in wt, k_6=0.3 s^{-1} and k_{11}=5 $\mu M^{-1} s^{-1}$). As a result, the rate of auxin biosynthesis provides a more important contribution to auxin concentration. Moreover, increase in k_6 increases auxin biosynthesis that is also controlled by ethylene and cytokinin. Experimentally, it has



been shown that *PLS* transcription does not affect ethylene biosynthesis (Chilley *et al*, 2006), and this result is confirmed by simulation (Figure 5E). Therefore, increase in the rate of auxin biosynthesis is predicted to be associated with PLS-induced decrease in cytokinin concentration. In addition, increase in k_6 also increases the auxin transport rate to the root, reflecting the negative effect of PLS on ethylene signalling (Chilley *et al*, 2006). Thus, for $k_{11} \neq 0\,\mu M^{-1}\,s^{-1}$ (in which case the ethylene response through its receptor is active), increase in auxin concentration by increase in k_6 is modelled to be due to the increase in both auxin biosynthesis and auxin transport.

The above analysis reveals that PLS exerts a control on how ethylene regulates auxin concentration at the root tip, by separately affecting auxin transport (in and out of the root) and auxin biosynthesis. It is clear that, although PLS interacts with ethylene signalling to regulate auxin concentration, it does not affect ethylene concentration (Figure 5E), as also evidenced by experiments (Chilley *et al*, 2006).

Known mutants relating to the interactions between k_6 and k_{11} can be analysed on the basis of Figure 5A, and Figure 5F shows some examples. The model is able to successfully reproduce three genotypes analysed by us previously (Chilley *et al*, 2006), namely *pls*, the *pls etr1-1* double mutant and the *PLS* overexpressing transgenic, *PLSox*. The model also predicts that for a *PLSox ETR1-1ox* genotype, auxin concentration should be similar to that in wild type. However, a *PLSox etr1-1* seedling would exhibit an increase in auxin concentration of 138% compared with the wild type. Chilley *et al* (2006) showed that auxin transport to, and accumulation in, the root are defective in *pls*. Similarly, the defective transport of auxin in *pls* is predicted using the model and agrees well with experimental observations (Figure 5D, the results for $k_{11}=5\,\mu M^{-1}\,s^{-1}$).

The relationship between CTR1 and PLS is analysed in Figure 5G. It is shown that, in a *ctr1* mutant background, increase in the level of *PLS* transcription (k_6) does not change the rate of auxin transport, but still increases the rate of auxin biosynthesis. As a result, auxin concentration in the root is predicted to increase as k_6 increases. The predicted auxin concentration for *PLSox/ctr1* is higher than that for *pls*, but lower than *PLSox*. For auxin concentrations of less than 0.256 μM (corresponding to the auxin concentration for *PLSox*), increase in auxin concentration increases root length (see Supplementary Figure S6 for details). Therefore, although *PLSox* increases root length compared with the *pls* mutant, or to a lesser extent, wild type, the *PLSox/ctr1* combination results in a much shorter root length than that in *PLSox*, but which is longer than that in the *ctr1* mutant. This demonstrates that the model, which assumes that the PLS peptide acts on the ethylene receptor, is able to experimentally explain observed results for the light-grown seedlings of *ctr1* and *PLSox/ctr1* mutants (Chilley *et al*, 2006). The modelling and experimental analysis for the relationship between CTR1 and PLS further confirms that component(s) downstream of ethylene signalling regulate auxin transport, and that PLS also regulates auxin biosynthesis.

Moreover, Figure 5G predicts that the change in auxin concentration (and therefore root length, see Supplementary information for details) for *ctr1*, when in a *PLSOx* background, is solely due to the change in the rate of auxin biosynthesis. To investigate this possibility experimentally, we grew *ctr1* seedlings for 5 days in the presence or absence of exogenous auxin (5 μM NAA), and observed that the root length of *ctr1* increased in the presence of auxin (Figure 6). This is consistent with the model prediction that the longer root in the *ctr1 PLSOx* seedlings is due to increased auxin accumulation compared with the *ctr1* root.

The above analysis shows that the mathematical model develops new insights into how PLS regulates auxin concentration by controlling the relative contribution of auxin transport and biosynthesis. Mutants can be reproduced and predicted. Experimental results describing the relationship between auxin concentration, exogenously applied ACC and the inhibition of ethylene responses can be understood in terms of their effects on the relative contribution of auxin transport and biosynthesis.

PLS expression and predictions on the response of the unknown molecule(s) X

The transcription of the *PLS* gene itself is regulated by both auxin and ethylene. Model analysis reveals that increase in

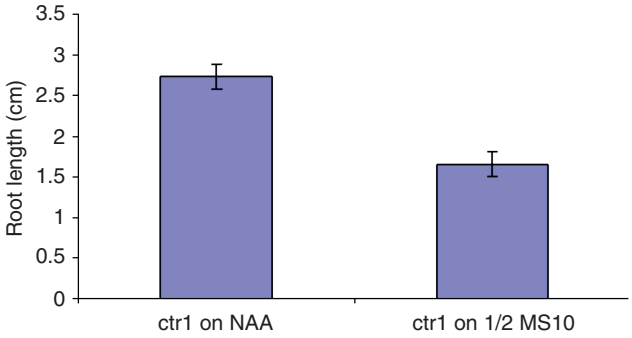


Figure 6 Primary root length of the *ctr1* mutant is increased by exogenous auxin (NAA). Roots of *ctr1* mutant seedlings (5 d.a.g.) grown on 0.05 μM NAA are longer than when grown on 1/2 MS10. Bars represent s.e.m. values, $n=15$.

Figure 5 PLS-mediated control of auxin concentration. (**A**) Effects of both *PLS* transcription and interaction of ethylene with its receptor on auxin concentration; (**B**) effects of both exogenous ACC and *PLS* transcription on auxin concentration. In *pls* mutant, auxin concentration decreases from 0.14 to 0.12 μM as exogenous ACC concentration increases from 0 to 10 μM. (**C**) Effects of both exogenous ACC and *PLS* transcription on auxin transport and biosynthesis; (**D**) effects of both *PLS* transcription and interaction of ethylene with its receptor on auxin transport and biosynthesis; (**E**) effects of *PLS* transcription on endogenous ethylene concentration; (**F**) filled bar: modelling results; unfilled bar: experimental measurements (Chilley *et al*, 2006). wt: $k_6=0.3\,s^{-1}$; $k_{11}=5\,\mu M^{-1}\,s^{-1}$; *pls*: $k_6=0.0\,s^{-1}$; $k_{11}=5\,\mu M^{-1}\,s^{-1}$. *pls etr1-1*: $k_6=0.0\,s^{-1}$; $k_{11}=0.03\,\mu M^{-1}\,s^{-1}$. PLSox: $k_6=1.0\,s^{-1}$; $k_{11}=5\,\mu M^{-1}\,s^{-1}$. PLSox ETR1-1ox: $k_6=0.45\,s^{-1}$; $k_{11}=10\,\mu M^{-1}\,s^{-1}$. PLSox *etr1-1*: $k_6=0.45\,s^{-1}$; $k_{11}=0.03\,\mu M^{-1}\,s^{-1}$. (**G**) Analysis of the effects of both *CTR1* and *PLS* transcription. Auxin concentration for total CTR1 concentration to be 0.3 μM (wt) is also included for comparison.

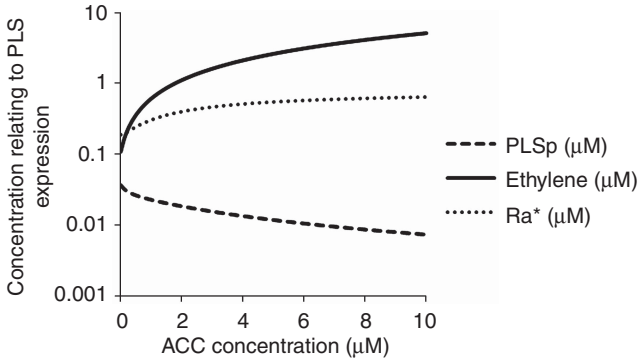


Figure 7 Increase in ACC increases both endogenous ethylene and the concentration of the activated form of auxin receptor, Ra*. Ethylene and Ra* contribute antagonistically to *PLS* expression, with the overall effect of increase in exogenous ACC is predicted to be the decrease in *PLS* expression.

exogenous auxin, which increases endogenous auxin concentration, increases *PLS* transcription (data not shown). This reflects the recognition that increase in auxin concentration increases the response of auxin signalling, and subsequently increases *PLS* expression (Casson *et al*, 2002). When ACC is exogenously applied, it also increases auxin concentration in wild type (Figure 5B), but it decreases *PLS* transcription (Figure 7). Model analysis shows that increase in ACC concentration increases both endogenous ethylene and the concentration of the activated form of auxin receptor, Ra*. Ethylene and Ra* contribute antagonistically to *PLS* expression, with the overall effect of increase in exogenous ACC is predicted to be the decrease of *PLS* expression (Figure 7). These trends qualitatively agree well with experimental observations (Casson *et al*, 2002; Chilley *et al*, 2006).

Although some components have been identified recently (Stepanova *et al*, 2008), the molecular basis for the interaction between the ethylene signalling response and the auxin response is not fully elucidated. A molecule or molecules, X, can be designated in the model as a module mediating the interaction. The model provides the opportunity to examine how the unknown molecule(s) X behave when exogenous auxin and ethylene concentrations change. Figure 8 shows that, when the exogenous ethylene concentration increases, the concentration of X initially increases rapidly, then approaches a constant value. However, when the exogenous auxin concentration increases, X initially decreases rapidly and then approaches a constant value. The trend analysis for X may be of use for searching for the candidate molecules that interact downstream of ethylene signalling to positively regulate the auxin response.

In addition, changes in root growth rate in response to exogenously applied hormones can be understood and predicted by combining modelling and experimental analysis (see Supplementary information for details).

Modelling and experimental analysis reveals a hormonal crosstalk circuit in *Arabidopsis*

The modelling and experimental analysis as described above reveals a hormonal crosstalk circuit in *Arabidopsis*, as summarised in Figure 9.

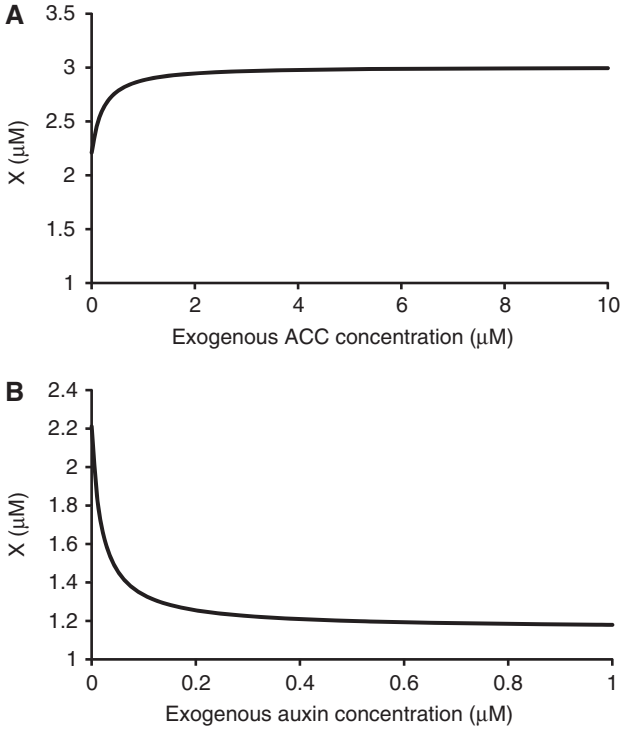


Figure 8 Prediction of response of unknown molecule, X, to exogenous ACC (**A**) and auxin (**B**) shows response of X to exogenous ACC and auxin follows opposite trends.

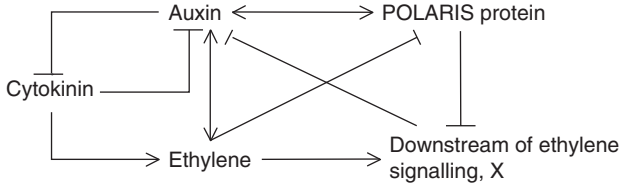


Figure 9 The hormonal crosstalk circuit revealed by modelling and experimental analysis in *Arabidopsis*.

Figure 9 shows that auxin, ethylene and cytokinin forms a tangled network that regulates their endogenous level; and *PLS* and unknown molecule(s), X, downstream of ethylene signalling, have roles in hormonal crosstalk. All known mutants can be understood using this crosstalk and can be quantified using the detailed interactions shown in Figure 1. Both Figures 1 and 9 provide a platform to further develop hormonal crosstalk circuit in *Arabidopsis*.

The current model concentrates on the study of the regulatory network for hormonal crosstalk. It is a single-cell model applied to root development. Therefore, the spatial distribution of hormones in the root cannot be addressed by the current model. However, on the basis of this model, by further including the spatial location of genes and by linking the fluxes described in this model with spatial location of those genes, a dynamic hormonal crosstalk model in spatial settings can be developed. Such a model should be able to develop insight into how hormonal interplay depends on the spatial expression of genes. It has been shown that model

development in a spatial setting is able to understand auxin distribution in root development (Grieneisen *et al*, 2007).

Discussion

Plant growth and development rely on appropriate signalling systems mediated by multiple hormones. We have, to the best of our knowledge, presented the first quantitative model to analyse the crosstalk between auxin, ethylene and cytokinin through the action of the *PLS* gene in *Arabidopsis*. We have then combined a modelling analysis with new experimental measurements based on predictions from the model. This combined approach has provided new insight into how PLS regulates auxin concentration in the root, by controlling the relative contribution of auxin transport into and out of the root and auxin biosynthesis in the root, and by integrating auxin, ethylene and cytokinin signalling.

Modelling and experimental analysis have revealed a hormonal crosstalk circuit in *Arabidopsis* (Figure 9). Moreover, model analysis reveals an interesting strategy for PLS in regulating hormonal crosstalk. The PLS protein confers flexibility on the regulation of endogenous auxin concentration by ethylene signalling (Figure 5A). According to the model, auxin concentration can be regulated by either the interaction of ethylene with its receptor, by *PLS* expression or both. As *PLS* expression itself is regulated by auxin, a regulatory loop between auxin, ethylene and *PLS* is formed through *PLS*. This loop flexibly regulates the endogenous auxin concentration/response that is to a significant extent responsible for root growth and development (Grieneisen *et al*, 2007).

Thus model analysis reveals that PLS exerts a control on how ethylene controls auxin concentration at the root tip by independently affecting auxin transport and auxin biosynthesis. On the one hand, increase in ethylene responses promotes auxin biosynthesis and inhibits *PLS* expression that also enhances auxin biosynthesis. However, increase in ethylene inhibits auxin transport into the root, and enhances auxin transport out, mediated by the action of PLS. If *PLS* is not expressed, ethylene no longer affects auxin transport and biosynthesis. Interestingly, due to the action of PLS, the dependence of auxin concentration on ethylene signalling can be flexible: auxin concentration may increase or decrease as ethylene responses increase, depending on parameters related to auxin biosynthesis and transport. Figure 2 shows that if the parameter for controlling auxin biosynthesis (the rate constant) is large, increase in ethylene signalling increases the auxin concentration as experimentally observed (Ruzicka *et al*, 2007; Swarup *et al*, 2007; Stepanova *et al*, 2007, 2008). However, if the parameter for controlling auxin biosynthesis is small, increase in ethylene signalling decreases auxin concentration. Figure 5C clearly shows that, when the parameter for controlling auxin biosynthesis is large and *PLS* is expressed, increase in auxin concentration occurs because increase in the auxin biosynthetic rate overtakes the decrease in auxin transport rate. Similarly, model analysis reveals that, when the parameter for controlling auxin biosynthesis is small, the decrease in auxin concentration is because increase in auxin biosynthesis rate cannot compensate for the decrease in auxin transport rate (data not shown). It is clear that the model is able to develop new insights into how PLS exerts controls on how ethylene affects auxin transport and auxin biosynthesis, and therefore controls auxin concentration at root tip.

The PLS protein is also predicted by the model to not to affect the endogenous concentration of ethylene (Figure 5E), although it affects ethylene signalling. This is in agreement with experimental evidence (Chilley *et al*, 2006). Therefore, modelling and experimental data suggest that PLS has a direct role in the transduction of ethylene signalling, rather than directly regulating the biosynthesis of ethylene. Our study also supports the view that auxin concentration is directly linked with the control of root length (Grieneisen *et al*, 2007).

On the basis of the model structure of Figure 1, the possible relationships between auxin biosynthesis pathway(s) and PLS-regulated hormonal crosstalk can be analysed. As shown in the Supplementary information, although the molecular basis for auxin biosynthesis is largely unknown, the regulation by PLSp of auxin biosynthesis must be realised through its compounded effects with ethylene and cytokinin: PLSp cannot regulate auxin biosynthesis independently of the regulation of ethylene and cytokinin. This demonstrates that different structures of this model may have different auxin concentration responses, revealing that the current combined modelling and experimental analysis is a powerful tool for dissecting the causal relationship for the interactions between genes and hormonal crosstalk.

Integration of biological knowledge into a mathematical model opens many channels to study the dynamics of a system with multiple hormonal signals. The current model concentrates on the action of the *PLS* gene product on crosstalk between auxin, ethylene and cytokinin. It clearly demonstrates that model analysis is able to explore how a cell perceives and transduces hormonal signals. Future model developments will include additional genes and analyse the role of gene–gene interactions in hormonal signalling. Moreover, development of a spatio-temporal model is able to assess how spatial-temporal dynamics of hormones and genes control plant development. Furthermore, in a changing environment, concentrations of hormones can be time dependent. How environment-dependent hormone concentrations affect plant growth and development can be assessed using a combination of mathematical modelling and experimentation.

Materials and methods

Modelling

The model is implemented in two simulators (COPASI (Hoops *et al*, 2006) and Berkeley Madonna (www.berkeleymadonna.com)). All computational results are exactly the same for both repositories. In COPASI repository, Deterministic (LSODA) method is used with an absolute tolerance of 1.0E12. In Berkeley Madonna, Rosenbrock (Stiff) method is used with a tolerance of 1.0E−10. Codes on both repositories can be made available on request (Junli.liu@durham.ac.uk). The system is able to establish a steady state within 50 s in general (See Supplementary information for details). When different mutants are analysed, we compare the steady-state results.

Growth and hormonal treatment of seedlings

The *pls* and *pls etr1* mutants of *Arabidopsis thaliana* have been described previously (Topping and Lindsey, 1997; Casson *et al*, 2002; Chilley *et al*, 2006). The *pls aux1* double mutant was generated by genetic crossing the *pls* promoter trap line with *aux1-7*, a kind gift of Professor Malcolm Bennett (University of Nottingham). *pls* DR5::GFP seedlings were also generated by crossing. In each case, F2 seedlings were analysed. For *in vitro* growth studies, seeds were stratified, surface-sterilized and plated on growth medium (half-strength Murashige and Skoog medium (Sigma, Poole, UK), 1% sucrose and 2.5% phytagel (Sigma) at 22 ± 2°C as described (Casson *et al*, 2009). For hormone application experiments, seeds were germinated aseptically on growth medium or growth medium containing hormones, as specified for each experiment. Cytokinin concentrations were determined by mass spectrometry according to Nordstrom *et al* (2004).

Microscopy

Confocal images were taken with a Bio-Rad Radiance 2000 microscope (Bio-Rad, Hemel Hempstead, UK) after counterstaining tissues with 10 mg/ml propidium iodide as described previously (Casson *et al*, 2009).

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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